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13. ABSTRACT ***(Maximum 200 Words)***
The subject of this research is "Functional Detection of p53 Mutations in Archival Prostate Cancer Tissue". The first aim of this research is to develop a yeast functional assay for the detection of individual p53 mutations in paraffin-embedded archival prostate cancer (CaP) tissues. The second aim is to determine the functional status of individual p53 mutations found in CaP. Although IHC and SSCP are commonly used to detect p53 mutations, their frequency is often overestimated by the former and underestimated by the latter techniques. In the first year, we have completed the evaluation of the application of this method. This includes optimizing the conditions for PCR and yeast transformation, measuring the background levels of the yeast functional assay of genomic p53 DNA, and comparing the yeast functional assay to the SSCP analysis of exon 5 of the p53 gene. Because the function of mutant p53 genes are so important to tumorigenesis and progression of cancer, these results will help in understanding the biological function of individual p53 mutations in CaP.

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Christopher P Evans

PI - Signature Date

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(5) Introduction

The subject of the research is "Functional Detection of *p53* Mutations in Archival Prostate Cancer Tissue". The **first aim** of this research is to develop a yeast functional assay for detection of individual *p53* mutations in paraffin-embedded archival prostate cancer (CaP) tissues. Although IHC and SSCP are commonly used to detect *p53* mutations, their frequency is often overestimated by the former and underestimated by the latter techniques. A new technique is needed to improve this detection rate. The yeast functional assay is such a technique which has been used to examine loss-of-function of *p53* mutations in other human cancers. We have used this technique to investigate the function of *p53* mutations in fresh-frozen CaP tissue. We will develop a strategy to permit us to study *p53* alterations in archival CaP tissue. To do this, we will use microdissection to enrich for tumor cells, thus permitting us to find *p53* mutations in small tumor foci. A critical part of our strategy will be to amplify exons 5, 6, 7 and 8 for genomic DNA obtained from paraffin embedded tissue followed by separately inserting them into plasmids to yield uniform *p53* products for each sample. The **second aim** is to determine the functional status of individual *p53* mutations found in CaP. We will examine the archival CaP tissues from at least 50 patients to find loss-of-function *p53* mutations by the yeast functional assay. We will also analyze dominant-negative effects by using a yeast-based system and gain-of-function mutations by cell transfection. Because these functions of mutant *p53* gene are important in tumorigenesis and progression of cancer, these results will help us to understand the biological function of individual *p53* mutations in CaP.

(6) Body (Studies and Results)

1. Development of the yeast functional assay of *p53* genomic DNA

We have developed a strategy to functionally detect individual *p53* mutations in paraffin-embedded archival CaP tissues. The strategy is based on the *Pfu*-mediated PCR amplification of *p53* from genomic DNA followed by yeast transformation **(Figure 1)**. We have optimized the analysis conditions, and these are described as follows.

I) Nested PCR amplification of individual exons 5-8 of *p53* gene

The individual tested exons were amplified by a two-step PCR approach **(Figure 1A)** using oligonucleotide primers positioned in the flanking introns (**Table 1**). The first-step of PCR was

Table 1. Primers for amplification of the tested exons of *p53* gene from genomic DNA

Exon	Outer pair	Nested pair
5	5'-GCTGCCGTGTTCCAGTTGC	5'-TACTCCCCTGCCCTCAACAA
	5'-TCAGTGAGGAATCAGAGGCC	5'-CATCGCTATCTGAGCAGCGC
6	5'-CTGGAGAGACGACAGGGCTG	5'-GTCTGGCCCCTCCTCAGCAT
	5'-TTAACCCCTCCCAGAGA	5'-CTCAGGCGGCTCATAGGGCA
7	5'-CTTGCCACAGGTCTCCCCAA	5'-GTTGGCTCTGACTGTACCAC
	5'-TCAGCGGCAAGCAGAGGCTG	5'-CTGGAGTCTTCCAGTGTGAT
8	5'-GTTGGGAGTAGATGGAGCCTGG	5'-TGGTAATCTACTGGGACGGA
	5'-GGCATTTTGAGTGTTAGACT	5'-CTCGCTTAGTGCTCCCTGGG

performed in 20 μl containing 15 pmol of each outer primer, 100 μM of each dNTP, 2 μl of 10X *Pfu* buffer, 0.75 unit of *Pfu* DNA polymerase and approximately 50 ng of genomic DNA. Amplification in an automated DNA thermal cycler was carried out by hot block start followed by 35 cycles of 95°C for 60 sec, 55°C for 60 sec and 72°C for 60 sec. Five μl of the first PCR product was digested with 1 unit exonuclease I at 37°C for 15 min to remove any remaining outer primers. The exonuclease I was then inactivated by incubating at 80°C for 15 min.

The second-step PCR was performed using 1 μl of the exonuclease-treated first-step product as the template in a 50 μl reaction. The reaction mixture contained the ingredients described above except for 10 pmol of each nested primer, 1.25 unit of *Pfu* DNA polymerase. The reaction was amplified by hot block start followed by 30 cycles, each consisting of 94°C for 30 sec, 55°C for 45 sec, and 72°C for 45 sec.

ii) Construction of *p53* fragment encoding codons 53-364.

The *p53* fragment encoding codons 53-364 were constructed by a two-step PCR method which fused each targeted *p53* exon from above with purified, complementary wild-type *p53* exons (**Figure 1B**). The wild-type *p53* exons were generated by *Pfu*-mediated PCR amplification of cloned wild-type *p53* cDNA using the primers listed in **Table 2**, so as to share terminal 20 base-pair overlaps with the tested exon. The first PCR was performed in 20 μl containing 2 μl of 10X *Pfu* buffer, 50 μM each of dNTPs, 0.2 μl of tested exon DNA (approximately 50 ng), 0.2 μl of each purified, complementary wild-type *p53* exon (approximately 50 ng), and 0.5 unit of *Pfu* DNA polymerase. The mixture was subjected to a hot block start and 15 thermal cycles, each consisting of 94°C for 60 sec, 55°C for 60 sec, and 72 °C for 120 sec. The second-step PCR was performed in 20 μl of volume containing 2 μl of 10X *Pfu* buffer, 2 μl of the first-PCR product, 1.25 units of *Pfu* DNA polymerase, 10 pmol each of primers P3 (5'-ATT TGA TGC TGT CCC CGG ACG ATA TTG AAC) and P4 (5'-ACC CTT TTT GGA CTT CAG GTG GCT GGA GTG), 2 μl of dimethyl sulfoxide (DMSO) and 50 μM dNTPs. The PCR reaction was carried out by a hot block start followed by 30 cycles of 94°C for 30 sec, 65°C for 60 sec and 78°C for 80 sec. The amplified *p53* products were examined by 1% agarose gel electrophoresis analysis of 5 μl of the PCR products.

Table 2. Primers for amplification of the wild-type exons of *p53* gene from cDNA

Exons	Primer sequences	Exons	Primer sequences
2-4	*5'-ATG GAG GAG CCG CAG TCA GAT** 5'-TTG TTG AGG GCA GGG GAG TA	**6-11**	*5'-TCA GTC TGA GTC AGG CCC TT* 5'-GCG CTG CTC AGA TAG CGA TG
2-5	*5'-ATG GAG GAG CCG CAG TCA GAT* 5'-ATG CTG AGG AGG GGC CAG AC	**7-11**	*5'-TCA GTC TGA GTC AGG CCC TT* 5'-TGC CCT ATG AGC CGC CTG AG
2-6	*5'-ATG GAG GAG CCG CAG TCA GAT* 5'-GTG GTA CAG TCA GAG CCA AC	**8-11**	*5'-TCA GTC TGA GTC AGG CCC TT* 5'-ATC ACA CTG GAA GAC TCC AG
2-7	*5'-ATG GAG GAG CCG CAG TCA GAT* 5'-TCC GTC CCA GTA GAT TAC CA	**9-11**	*5'-TCA GTC TGA GTC AGG CCC TT* 5'-CCC AGG GAG CAC TAA GCG AG

* The *italic* primer sequences are identical in each column.

iii) Yeast transformation.

The yeast functional assay of PCR-constructed *p53* fragments was performed following Flaman's method (**1**). Plasmids pLS76 and pRDI-22, and a yeast strain yIG397 which contains an integrated plasmid with the *ADE2* gene under the control of a p53-responsive promoter, were graciously provided by Dr. Richard Iggo (Oncogene Group, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). The plasmid pRDI-22 was digested using *Hind*III and *Stu*I to create a gap between codons 67 and 347 of the wt *p53* insert. The gapped pRDI-22 was co-transformed into yeast with the fused *p53* products that span codons 53 through 364, using lithium acetate-polyethylene glycol (LiOAc-PEG). The transformed yeast cells were plated on a dish containing synthetic minimal medium minus leucine plus 5 µg/ml of adenine, and grown for 3 days at 35°C. Dishes then were stored at 4°C for 2-4 days to enhance color development. Transactivational function is scored based on the color of yeast colonies. Large white colonies indicate wild-type transactivational function; small red colonies, resulting from limited growth due to the accumulation of intermediate products in adenine metabolism pathway, are scored as total loss of function; and pink colonies indicate partial function (**Figure 1C**). More than 200 yeast colonies were counted for each transformation, and the percentages of white, red and pink colonies were calculated.

2. Yeast Functional Assay of Wild-type Tested *p53* Exons

In order to examine if construction of *p53* fragment will increase the background levels of red or pink yeast colonies, we first tested the wild-type *p53* exons 5, 6, 7 and 8 that were amplified from wild-type genomic DNA. These individual exons were amplified separately and linked to the wild-type exons to generate the *p53* fragments encoding codons 53-364. After yeast transformation, the percentage of white, red and pink yeast colonies were calculated. The assay was repeated twice and the results were listed in Table 3. From the two assays of exons 5-8, the levels of red background were 3-7% and the pink background level was zero. Previous studies have shown that the background levels of red yeast colonies were 5-10%. Thus, these results in Table 3 demonstrates that the yeast functional assay of *p53* genomic DNA developed in this research has low background levels for exons 5-8.

Table 3. Yeast function assay of individual p53 exons amplified from wild-type genomic DNA

Exon	% Yeast colonies (assay 1)			% Yeast colonies (assay 2)		
	white	red	pink	white	red	pink
5	96	4	0	96	4	0
6	93	7	0	93	7	0
7	93	7	0	93	7	0
8	96	4	0	97	3	0

3. Yeast Functional Assay of *p53* Mutations from Archival CaP Tissues

Eleven selected CaP samples were used to test the yeast functional assay of *p53* genomic DNA. The area within the tissue block which contained the region of pathologic interest was marked

by a pathologist. Histologic enrichment was performed on paraffin-embedded archival CaP tissue. Tumor tissue dissected from 50 micron sections was deposited in 1.5 ml microcentrifuge tubes, deparaffinized with xylene, rinsed with 100% ethanol, and dried. The resulting tissue was suspended in 250 µl of lysis buffer (50 mM Tris and 5 mM EDTA, pH 8), and 25 µl of the proteinase K solution was added. Incubation was done for 48 hours at 42°C with the addition of 25 µl of the proteinase K solution every four hours during the day. DNA was extracted with phenol:chloroform once and chloroform once, precipitated with ethanol, dried and dissolved in 20 µl TE buffer. The resulting genomic DNA was used for PCR amplification. The resulting individual *p53* exons were then used for SSCP analysis and yeast functional assay.

For these 11 archival CaP tissues, we have completed both the SSCP analysis and yeast functional assay of exons 5 and 6. Three *p53* mutations in exon 5 were detected by the SSCP analysis and four by yeast functional assay. No mutation was detected in exon 6. The exon 5 of *p53* gene from these positive specimens were sequenced. For yeast colony sequencing, three single yeast colonies were separately digested with zymolyase. The *p53* expression plasmids were isolated and transformed into an *E.coli.* strain. The plasmids were then recovered, purified and sequenced on an ABI Prizm 377XL sequencer. These results from both SSCP and yeast assay are listed in **Table 4**. The identical mutations were found in two specimens by the SSCP analysis and the yeast functional analysis (**Figure 2**). These results show that yeast functional assay of genomic *p53* mutations is more sensitive than the SSCP analysis.

Table 4. Yeast functional assay of Exons 5 of genomic *p53* gene in archival CaP tissues

№	SSCP/Sequencing	Yeast Functional Assay			Sequencing of Yeast Colonies
		white	red	pink	
1	E5:158, CGC→TGC, Arg→Cys	85%	11%	**4%***	E5:158, CGC→TGC, Arg→Cys
2	E5:179, CAT→CTT, His→Leu	67%	**33%**	0%	E5:179, CAT→CTT, His→Leu
3	E5126, TAC→TGC, Tyr→Cys	82%	**18%**	0%	E5:152, CCG→TCG, Pro→Ser
4	undetectable	54%	**45%**	1%	E5:179, CAT→CGT, His→Arg

* The bold numbers mean that these specimens contain p53 mutations in exon 5.

(7) Key Research Accomplishments

1. The primers required in this research have been designed and synthesized.
2. The methods of microdissection and PCR conditions have been optimized.
3. The yeast functional assays of genomic *p53* DNA have been developed.
4. The analysis of the wild-type exons 5, 6, 7 and 8 of *p53* gene have been performed.
5. The analysis of the mutant exons 5 and 6 of *p53* gene have been performed.

(8) Reportable Outcomes

1. A new method of evaluating archival prostate cancer tissue for functionality mutant *p53* alleles. Di Mauro SM, Shi XB, deVere White RW, Evans CP. Presented at 5th Cancer Research Symposium. University of California Davis, Cancer Center, Sept 17-18, 1999, Sacramento.
2. The identification of functionally mutant p53 alleles in archival prostate cancer tissue. Di Mauro SM, Shi XB, deVere White RW, Evans CP. Presented at the Western Section AUA, Sept 28, 1999.

(9) Conclusions

In the first supported year of this research, we have developed a new technique that permit us to study *p53* mutations in archival CaP tissue. We have completed the evaluation of the application of this method, including optimizing the conditions for PCR and yeast transformation, measuring the background levels of yeast functional assay of genomic *p53* DNA, and comparing the yeast functional assay to the SSCP analysis of exon 5 of *p53* gene. It has found that the developed method can be used to functionally detect the *p53* mutations in archival CaP tissues and it is more sensitive than SSCP in detection of *p53* mutations. We will use the technique to identify the functional mutations of *p53* gene from a large group of archival CaP and bladder cancer tissue in the 2nd year.

(10) References

1. Flaman, J. M., Frebourg, T., Moreau, V., Charbonnier, F., Matyin, C., Chappuis, P., Sappino, A. P., Limacher, J. M., Bron, L., Benhattar, J., Tada, M., van Meir, E. G., Estreicher, A., and Iggo, R. D. A simple p53 functional assay for screening cell lines, blood, and tumors. Proc. Natl. Acad. Sci. USA, *92:* 3963-3967, 1995.

(11) Appendices

Figure 1: PCR-based yeast functional detection of p53 mutations in archival prostate cancer tissues.

Figure 2: p53 mutations in exon 5 detected by SSCP analysis and yeast functional assay.

Figure 1. PCR-based yeast functional detection of *p53* mutations in archival prostate cancer tissues

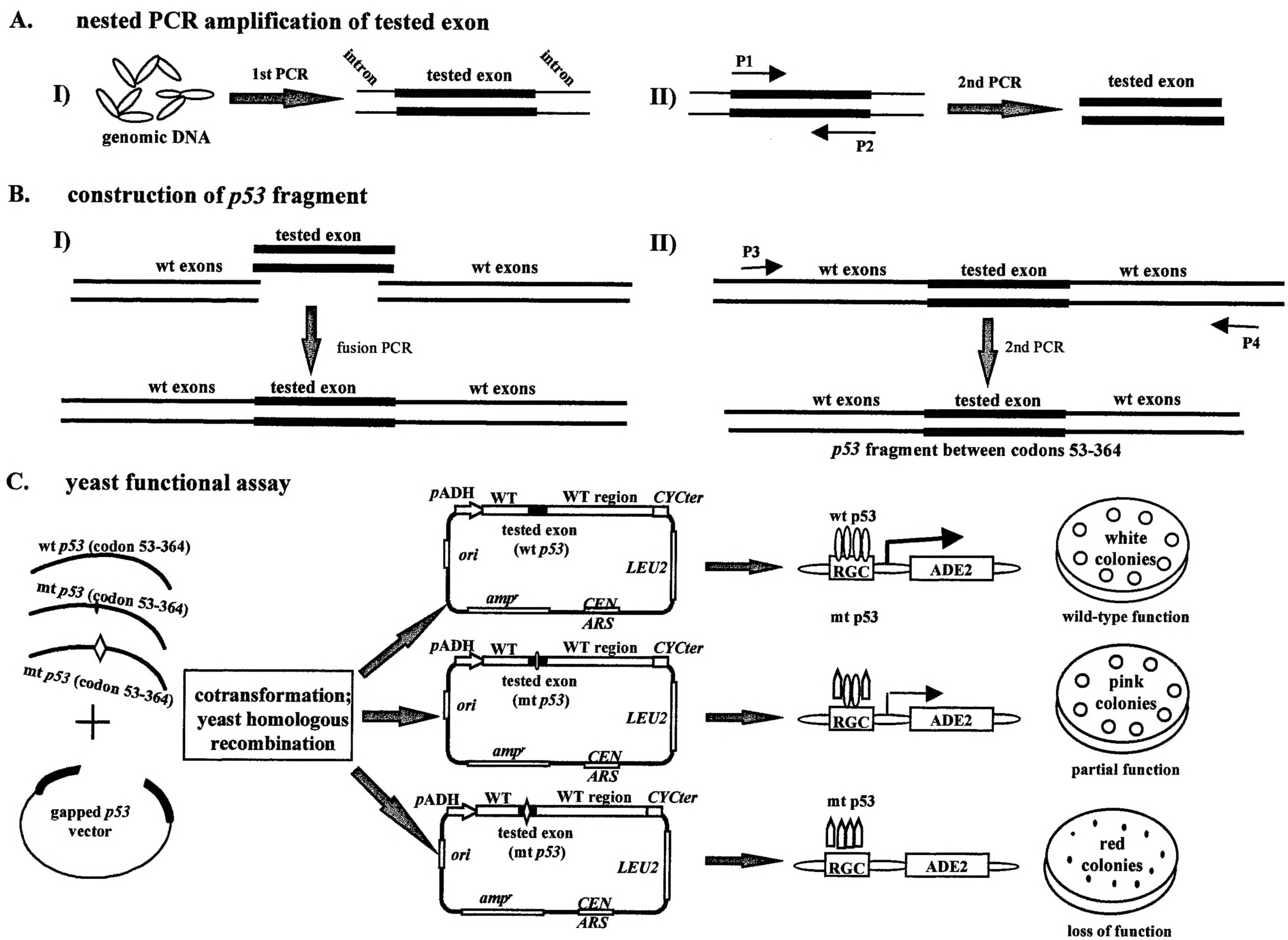

Figure 2. ***p53*** **mutations in exon 5 detected by SSCP analysis and yeast functional assay**